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Early Development of Zooxanthella-Containing Eggs of the Corals *Pocillopora verrucosa* and *P. eydouxi* with Special Reference to the Distribution of Zooxanthellae

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Abstract. Some hermatypic corals spawn eggs that contain zooxanthellae. We followed development of zooxanthella-containing eggs of two such species, *Pocillopora verrucosa* and *P. eydouxi*. We also documented changes in the distribution pattern of zooxanthellae during development. Oocytes of both species took up zooxanthellae 3 to 4 days before spawning. At first, zooxanthellae were evenly distributed in oocytes, but they later moved to the hemisphere that contained the germinal vesicle. After fertilization, early cleavage events were holoblastic, progressing by furrow formation. The first cleavage furrow started at the hemisphere that contained zooxanthellae, dividing the zooxanthellate complement of the zygote about equally into the two blastomeres. The second division divided each blastomere into one zooxanthellae-rich cell and one with few zooxanthellae. With continued cell division, blastomeres containing zooxanthellae moved into the blastocoel. The blastocoel disappeared at about 5 h after the first cleavage, and the central region of the embryo was filled with cells containing either zooxanthellae or lipid droplets, forming a stereogastrula. Our results suggest that only blastomeres that had been determined to develop into gastrodermal cells receive zooxanthellae during cleavage. This determination appears to take place, at the latest, by the second cell division at the four-cell stage.

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Introduction

Reef-building corals harbor intracellular symbiotic dinoflagellates, zooxanthellae, in their endodermal cells. Some hermatypic corals acquire their symbionts from their mother colony before fertilization (Kojis and Quinn, 1981; Babcock and Heyward, 1986; Tomascik and Sander, 1987; Yeemin, 1988; Glynn *et al.*, 1991, 1994; Heyward *et al.*, 1987; Kinzie, 1993, 1996; Sier and Olive, 1994; Kruger and Schleyer, 1998). It is not known how zooxanthellae are delivered to oocytes and how their distribution relates to their eventual restriction to the endodermal cells in adults. Early development of scleractinian corals has been described in various species (*e.g.*, Szmant-Froelich *et al.*, 1980, 1985; Babcock and Heyward, 1986; Harrison and Wallace, 1990). However, early development of corals with oocytes containing zooxanthellae has been described only in the spawning species *Montipora effusa* (Yeemin, 1988) and *M. verrucosa* (Maté *et al.*, 1998) and the brooding species *Porites porites* (Tomascik and Sander, 1987).

Although zooxanthellae are generally restricted to the gastrodermis of adult corals, they are at least temporarily observed in the ectoderm of planulae of some corals and soft corals (Szmant-Froelich, 1985; Benayahu *et al.*, 1988; Benayahu, 1997; Benayahu and Schleyer, 1998; Schwarz *et al.*, 1999). This is probably because infection first occurred in the ectoderm cells of embryos or early planulae (Szmant-Froelich *et al.*, 1985) or because dividing cells of these stages transferred the multiplying symbionts to their daughter cells, including presumptive ectoderm cells (Benayahu, 1997; Benayahu and Schleyer, 1998). In these cases, zooxanthellae were transferred from ectoderm to endoderm

across the mesoglea before larvae develop into mature planulae (Benayahu, 1997; Benayahu and Schleyer, 1998). Montgomery and Kremer (1995) also found that in the larvae of a scyphozoan, *Linuche unguiculata*, the algae were found mostly in the ectodermal cells, and suggested mechanisms by which zooxanthellae could be transferred from ectoderm to endoderm of planulae.

The corals *Pocillopora eydouxi* and *P. verrucosa* release zooxanthellate eggs, which display an uneven distribution of algal cells (Hirose *et al.*, unpubl. data). It is likely that, in these corals, zooxanthellae are not equally delivered to all daughter cells but go more or less exclusively to presumptive endoderm cells. If zooxanthellae become restricted to endoderm cells during the course of development, the larvae do not need to transfer the algae from ectoderm to endoderm as described in the soft corals (Benayahu, 1997).

In the present study, we followed early development of zooxanthellate eggs of the corals *P. eydouxi* and *P. verrucosa*. We studied changes in the distribution pattern of zooxanthellae during early development of the corals to determine mechanisms by which the distribution of zooxanthellae becomes localized to the endoderm of planulae.

Materials and Methods

Branches, 7–12 cm long, were collected from colonies of *Pocillopora verrucosa* a few days before the new moon and from *P. eydouxi* a few days before the full moon in June and July 1998. Colonies were collected from reefs at Sesoko Island, Okinawa. The branches were placed separately into 3-l plastic containers supplied with unfiltered running seawater. The hermaphroditic colonies of *P. verrucosa* and *P. eydouxi* spawned gametes for about 30 min in the early morning a few days after the new moon, and a few days after the full moon, respectively (Kinzie, 1993; Hirose *et al.*, unpubl. data). Both species first released sperm and then negatively buoyant eggs. To collect gametes, the supply of seawater was stopped before the expected spawning time, about 0630 h. After sperm had been shed, they were collected by sucking up seawater from the container in a large plastic pipette. The pipette was rinsed with a diluted hypochlorite solution and then with seawater to avoid contamination of gametes. Released eggs were collected by pipette from the bottom of the container and placed in a plastic beaker. Eggs were fertilized by mixing released gametes (100–300 ml suspension each) from two or three colonies in a plastic beaker. Filtered (0.45 μm) seawater was added to the beaker to make the final volume to 1 or 2 l. Fertilized eggs were kept in the seawater at a room temperature 28°–30°C. Eggs and embryos were sampled and observed under a light microscope at intervals of from 30 min to 1 h, and photomicrographs were taken with a microscope equipped with an epifluorescent system (Nikon Microphot).

Histology and transmission electron microscopy

Eggs and embryos were collected in a microtube and allowed to settle to the bottom. The supernatant was then discarded and fixative added. The specimens were fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) containing 3% NaCl for 2 h or more. The specimens were rinsed in the same buffer three times and post-fixed in 1% osmium tetroxide in the same buffer for 1 h on ice, dehydrated in a graded series of acetone, immersed in *n*-butyl glycidyl ether (QY1), and embedded in Spurr's resin. For light microscopic observation, sections 0.5–1 μm thick were stained with 1% methylene blue-1% azur II in 1% borax. For electron microscopy, silver to gold sections were stained with uranyl acetate and lead citrate and observed under a JEOL JEM-2000EX electron microscope at an acceleration voltage of 100 kV.

Results

Oocytes took up zooxanthellae 3–4 days before spawning in both species. Zooxanthellae were first distributed evenly in the ooplasm (Fig. 1A, B), but later, 1–2 days before spawning, the algae became concentrated in the hemisphere that contained the germinal vesicle. The other hemisphere contained many lipid droplets of about the same size as the zooxanthellae. Although the germinal vesicle was no longer apparent by the time the eggs were spawned, the zooxanthellae remained concentrated in the hemisphere of the egg that contained the nucleus (Fig. 1C, D). At spawning, eggs of both species were about 140 μm in diameter and contained about 130 zooxanthellae (Hirose *et al.*, unpubl. data).

Cleavage took place by progressive furrow formation (Fig. 1E), at intervals of 30 to 40 min. The first cleavage furrow started at the hemisphere that contained the zooxanthellae and the oocyte nucleus, dividing the zooxanthellae equally into two blastomeres, each with a roughly equal complement of zooxanthellae still concentrated in the hemisphere containing the nucleus (Fig. 1F). At the second cleavage, each blastomere was divided into one zooxanthellae-rich blastomere and one with few zooxanthellae (Fig. 2A). By the 64- to 128-cell stage, some blastomeres in the morula contained one or sometimes more zooxanthellae, while others contained none (Fig. 2B). As cleavage proceeded, a blastocoel formed and blastomeres with zooxanthellae moved into this space (Fig. 2C, D). The outer layer of the blastula consisted of relatively large blastomeres containing no zooxanthellae. At about 5 h after fertilization, blastomeres containing zooxanthellae, lipid droplets, or both filled the blastocoel, resulting in a stereogastrula (Fig. 2E, F). Gastrulation appeared to occur by delamination rather than by invagination.

Blastomeres in the outer layer had microvilli on their outer surface and characteristic granules just below the cell membrane (Fig. 3A). Blastomeres in the outer layer were

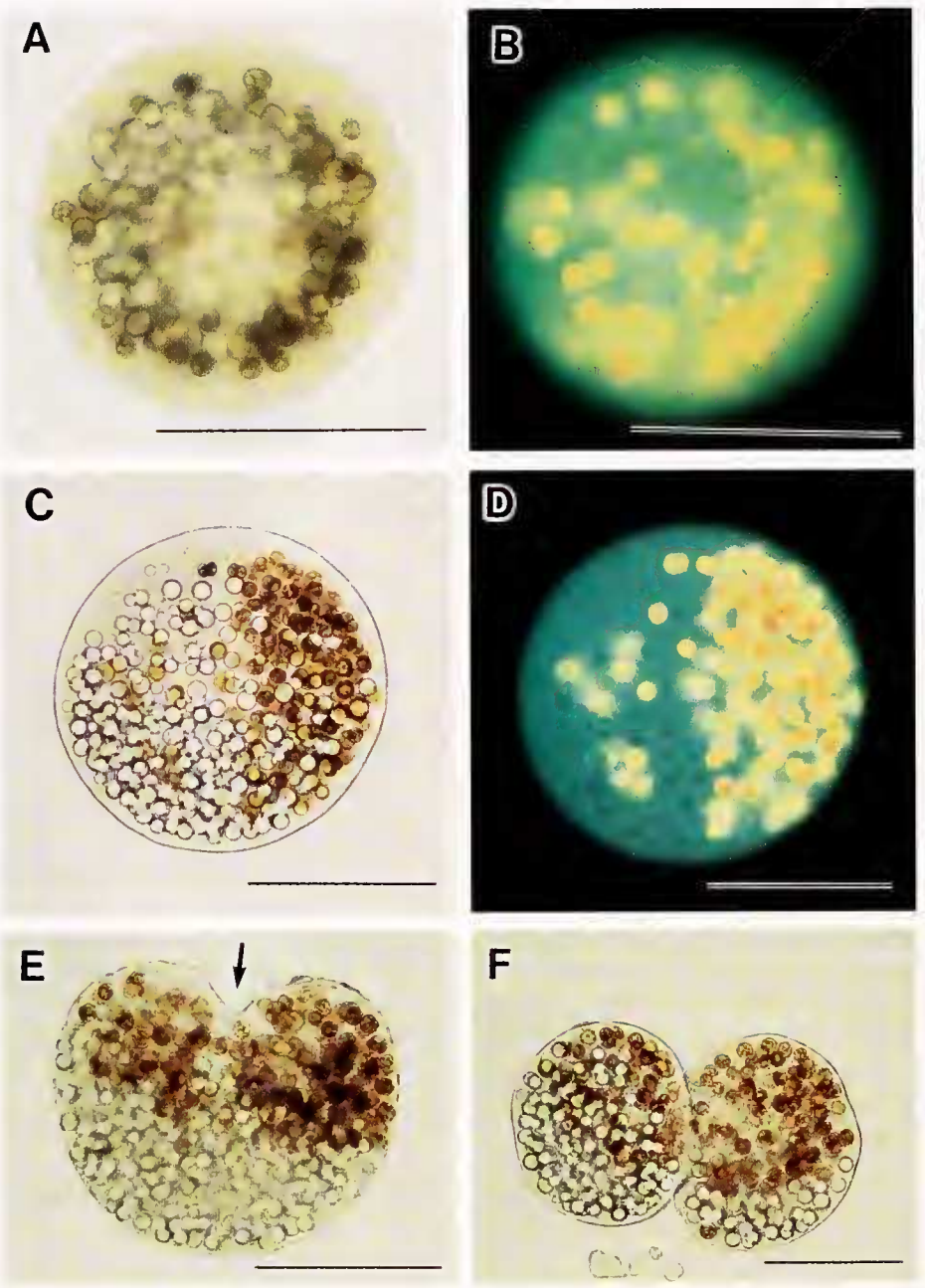

Figure 1. Early development of *Pocillopora verrucosa:* from unfertilized egg to two-cell stage. (A) Oocyte isolated from the gonad. Zooxanthellae are distributed evenly in the cytoplasm. The germinal vesicle is at the center of the oocyte. (B) Oocyte viewed under epifluorescence (BV excitation). The red fluorescence is due to algal chlorophyll. Cytoplasm of the oocyte exhibits blue-green autofluorescence. (C) Spawned egg. Zooxanthellae are mainly located in the right hemisphere and lipid droplets in the left hemisphere. (D) The same egg, observed under epifluorescence (BV excitation). (E) First cleaving stage. Cleavage furrow (arrow) starts at the hemisphere that contains the zooxanthellae. (F) Two-cell stage. Zooxanthellae are divided equally into the two blastomeres. Bars = 100 μm.

connected to each other by the contact junctions near the apical surface (Fig. 3B). In other regions of the interface, blastomeres were only loosely attached to each other or were separated by extracellular space. Villi-like cellular processes were observed in the extracellular space, and those from neighboring blastomeres were often intermingled (Fig. 3C). When blastomeres in the outer layer contained zooxanthellae, the zooxanthellae were usually at the lower or lateral margin of the blastomere. Zooxanthellae at the lateral margin of the blastomere bulged into the extra-

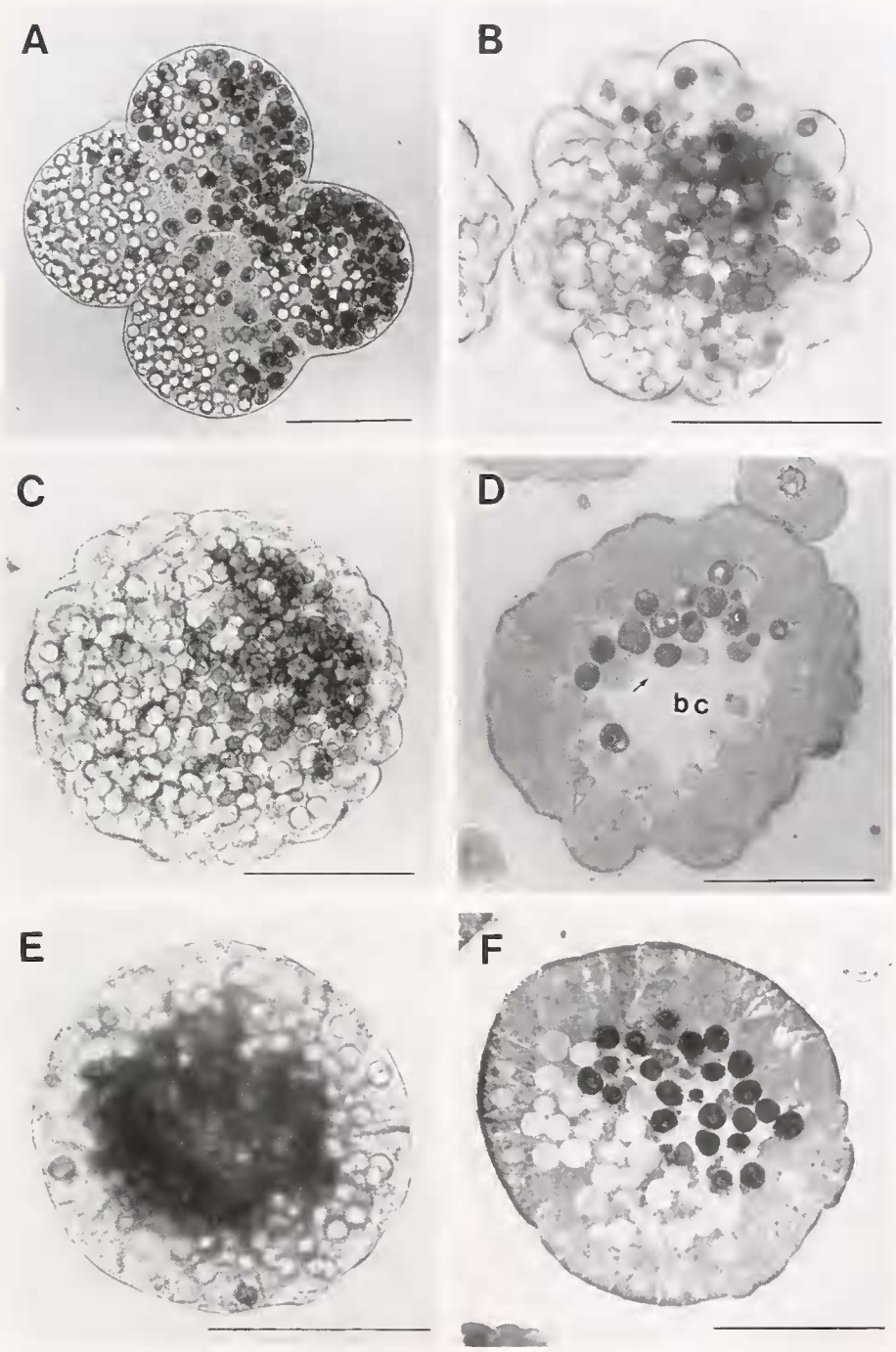

Figure 2. Early development of *Pocillopora verrucosa:* four-cell stage to gastrula. (A) Four-cell stage. The second cleavage plane was normal to the first cleavage plane, thus dividing the blastomere into a zooxanthellae-rich blastomere and a lipid-droplet-rich blastomere. (B) Morula-stage embryo. Blastomeres are round; some contain a single zooxanthellae. (C) Blastula. Zooxanthellae are still restricted to one hemisphere. (D) Section of a blastula. Blastomeres containing one or more zooxanthellae (arrow) and those containing lipid droplets are located in the blastocoel (bc), while the surface layer is composed of larger blastomeres with no algae. (E) Gastrula. The center of the gastrula appears dark due to accumulation of zooxanthella-containing blastomeres. (F) Section of a gastrula. Blastomeres containing zooxanthellae and those containing lipid droplets fill the inner space of the gastrula, forming a stereogastrula. Bars = 100 μm.

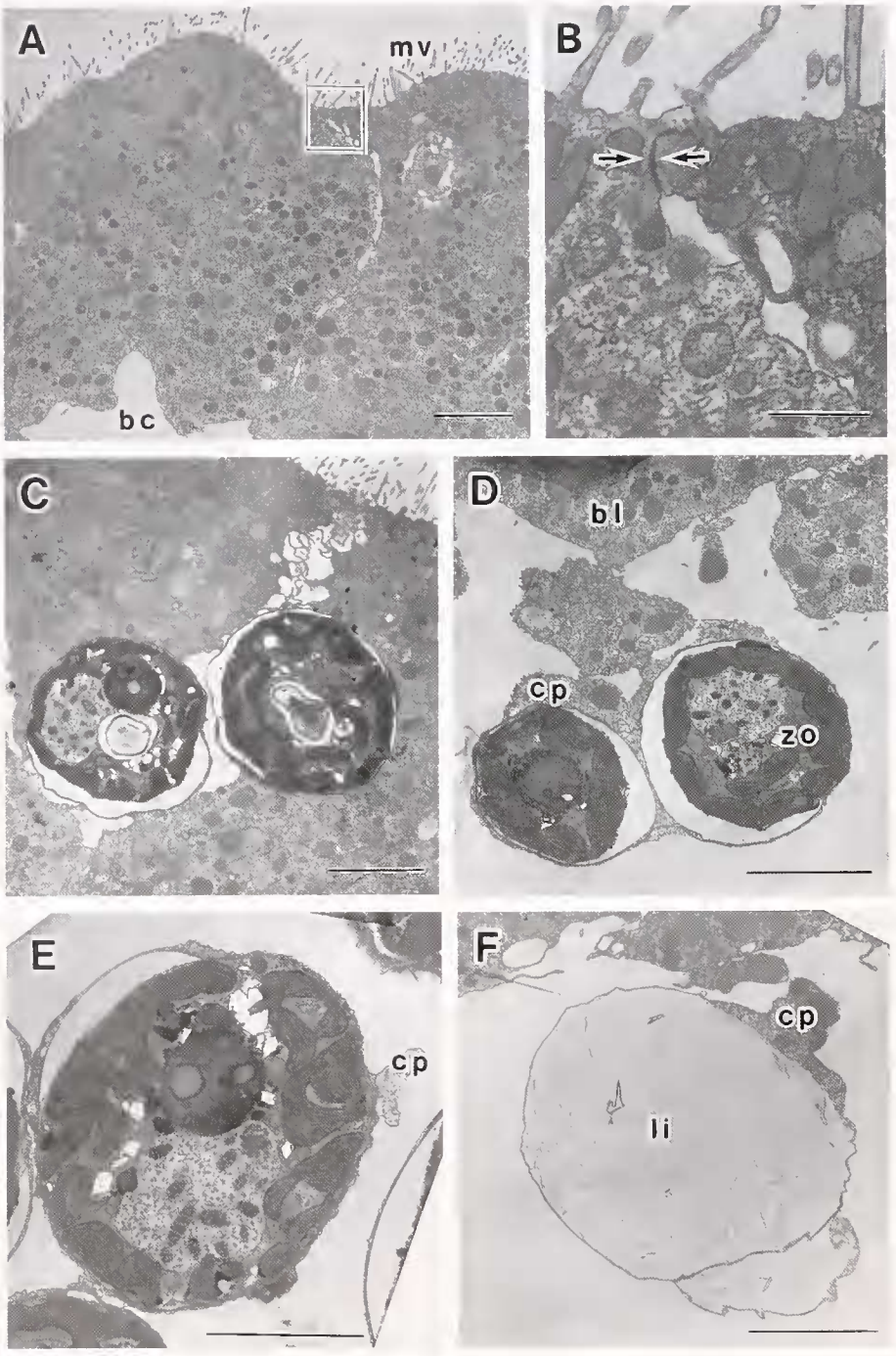

Figure 3. Electron micrographs of an early gastrula of *Pocillopora verrucosa*. (A) Blastomeres in the outer layer of an embryo. (B) Higher magnification of the boxed area in (A), showing contact junction near the apical surface (arrow). (C) Blastomere in the outer layer containing zooxanthellae. Zooxanthellae bulge into the neighboring blastomere. (D) Two zooxanthellae in a cellular process, which appears to be still connected to the outer layer blastomere. (E) Zooxanthellae surrounded by a small amount of host cytoplasm. (F) Lipid droplet surrounded by a small amount of cytoplasm. bc = blastocoel, bl = blastomere, cp = cytoplasm, li = lipid droplet, mv = microvilli, zo = zooxanthella. Bars = 5 μm except in (B), where bar = 1 μm.

cellular space and sometimes into neighboring blastomeres (Fig. 3C). Similarly, zooxanthellae or lipid droplets located at the lower margin of the blastomeres bulged into the blastocoel. In such cases, a constriction was often observed between the central cytoplasm and the protrusion containing a zooxanthella or a lipid droplet (Fig. 3D). Most zooxan-

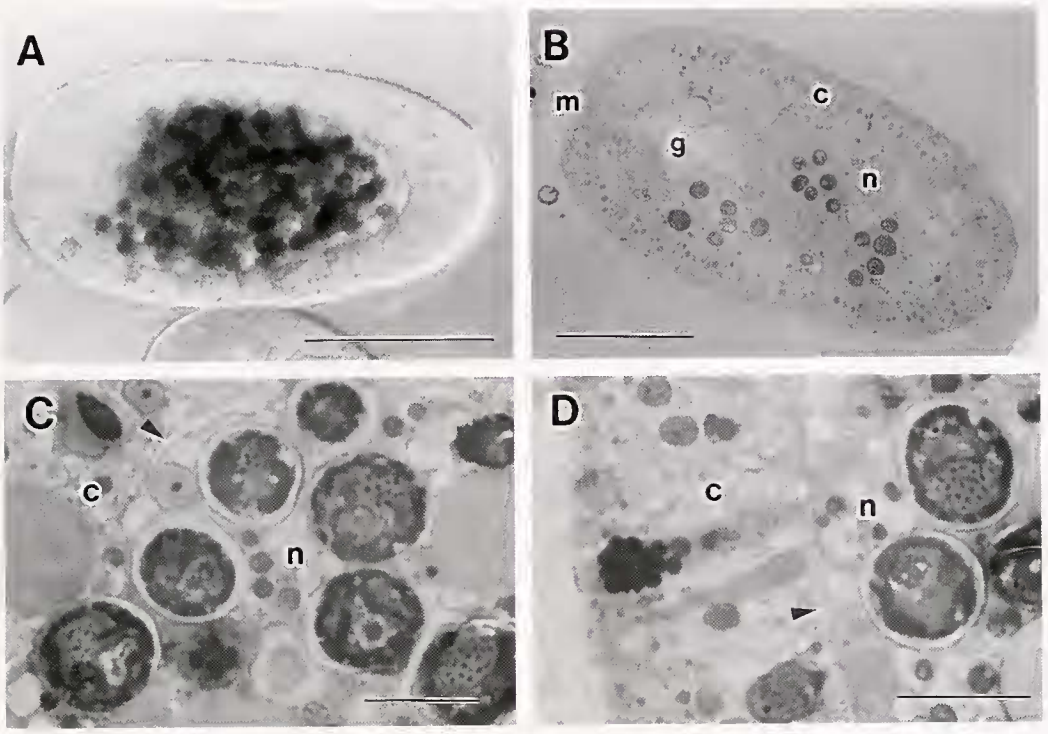

Figure 4. Planula of *Pocillopora verrucosa* 24 h after fertilization. (A) Photomicrograph of a fixed planula taken under differential interference optics. The planula is completely ciliated at this stage. (B) Histological section of a planula. Zooxanthellae and lipid droplets are in the endodermal cells. (C–D) Photomicrographs of the body wall of a planula taken under oil immersion. The ectoderm and endoderm are clearly separated by the mesoglea (arrowhead). The ectoderm consists of columnar cells. c = ectoderm, g = gastrovascular cavity, m = mouth opening, n = endoderm. Bars = 100 μm in (A) and (B), 20 μm in (C) and (D).

thellae and lipid droplets in the blastocoel were surrounded by a small amount of cytoplasm and appeared to be free—that is, detached—from blastomeres in the outer layer (Fig. 3E, F).

Spherical embryos with a smooth surface as shown in Figure 2E and F were observed 6 h after fertilization. Ciliated larvae started to swim 8 h after fertilization. The embryos became elliptical and swam spirally by 9–10 h after fertilization. An oral pore was formed by invagination of the epidermis, and a gastrovascular cavity was formed as gastrodermal cells became organized 24 h after fertilization (Fig. 4A, B). At this stage the ectodermal layer—the planula's epidermis—consisted of characteristic columnar cells, and the epidermis and gastrodermis were separated by distinct mesoglea (Fig. 4C, D). Embryos at this stage were typical planulae. Generally, only gastrodermal cells contained zooxanthellae, though a few zooxanthellae were observed in ectoderm of some planulae. Planulae 48 h old possessed some nematocysts.

Discussion

Although early development of scleractinians has been described (*e.g.*, Szmant-Froelich *et al.*, 1980, 1985; Babcock and Heyward, 1986; Harrison and Wallace, 1990), this is the first report describing the processes by which zooxanthellae become restricted to the endoderm during the course of embryogenesis. In the two *Pocillopora* species studied, regions of egg cytoplasm are differentiated and cell fates are apparently decided early in development, possibly before fertilization. Zooxanthellae moved toward the animal pole 1–2 days before spawning. The first cleavage apportioned zooxanthellae more or less equally between the first two blastomeres. At the second cleavage, however, two of the four blastomeres received almost all the zooxanthellae, while the other two had few or none. This uneven distribution of zooxanthellae persisted until the zygotes developed into gastrulae.

As cleavage progressed, relatively large blastomeres without zooxanthellae came to occupy the outer layer of the embryo as the blastocoel opened. Later, blastomeres containing zooxanthellae or lipid droplets detached from the outer layer and dropped into the blastocoel until it was filled with blastomeres containing zooxanthellae and lipid droplets. In these two species of *Pocillopora*, gastrulation may occur due to delamination rather than invagination, resulting in a stereogastrula. Gastrulation through delamination

has been suggested for *Astrangia danae* (Szmant-Froelich *et al.*, 1980), *Favia fragum* (Szmant-Froelich *et al.*, 1985), and *Montipora verrucosa* (Maté *et al.*, 1998).

Titlyanov *et al.* (1996, 1998) observed degraded zooxanthellae in planulae as well as in adult polyps of hermatypic corals and suggested that digestion of zooxanthellae occurs both in planulae and in adult polyps. We saw no such degraded zooxanthellae in the surface layer of embryos or early planulae. If zooxanthellae are not digested during early development, they must be transferred from blastomeres that are determined to develop into symbiont-free ectodermal cells to blastomeres that are fated to develop into algae-bearing endodermal cells. This ontogenetic redistribution of algae might occur in several ways. One possibility is that zooxanthellae move basally within blastomeres so that subsequent horizontal cell division results in surface ectodermal cells and centrally located endodermal cells that contain zooxanthellae. Our observations suggest that zooxanthellae, along with small amounts of cytoplasm, were separated from surface cells and dropped into the blastocoel. This process is similar to the "pinching off" suggested for the transfer mechanism of zooxanthellae from follicle cells to oocytes and from ectoderm to endoderm in some soft corals (Benayahu, 1997; Benayahu *et al.*, 1992; Benayahu and Schleyer, 1998). However, the small "blastomeres" containing zooxanthellae (Fig. 3E, F) could also be produced by unequal division rather than by pinching off. If this were the case, there should be animal nuclei in these structures. These basally derived cells would then develop into gastrodermal cells. Another possibility is that ectodermal cells expel zooxanthellae by exocytosis and adjacent endodermal cells take them up by phagocytosis. The observation that zooxanthellae within vacuoles of the blastomere in the outer layer often protruded to the intercellular space, bulging into the neighboring cell, suggests that this possibility cannot be ruled out.

Few if any zooxanthellae were found in the outer layer of gastrulae or the ectoderm of planulae of the two *Pocillopora* species studied. However, zooxanthellae are sometimes found in the ectoderm of early planulae of some corals (*Favia fragum:* Szmant-Froelich *et al.*, 1985; *Fungia scutaria:* Schwarz *et al.*, 1999), soft corals (*Xenia umbellata:* Benayahu *et al.*, 1988; *Litophyton arboreum:* Benayahu *et al.*, 1992; Benayahu, 1997; *Anthelia glauca:* Benayahu and Schleyer, 1998), and the scyphozoan *Linuche unguiculata* (Montgomery and Kremer, 1995). It has been suggested that, in the early developmental stages, zooxanthellae show no specificity towards presumptive endodermal cells (Benayahu, 1997; Benayahu and Schleyer, 1998). However, as planulae develop, zooxanthellae are found increasingly in the endoderm and eventually become restricted to the gastrodermis of polyps. Several mechanisms by which the algae are translocated from ectoderm to endoderm have been suggested (Montgomery and Kremer, 1995; Benayahu, 1997; Benayahu and Schleyer, 1998). Montgomery and Kremer (1995) suggested that ectoderm cells infected by zooxanthellae may migrate to the endoderm of planulae. Benayahu (1997) and Benayahu and Schleyer (1998) observed that, in the soft corals they studied, zooxanthellae pass through temporarily opened gaps in the mesoglea towards the endoderm. Throughout the process, each zooxanthella resides within a vacuole in the detached ectodermal cytoplasm. However, we did not observe such a transfer of zooxanthellae from ectoderm to endoderm in planulae of the two *Pocillopora* species. In these corals, zooxanthellae appeared to be transferred more or less exclusively to blastomeres that were fated to develop into endodermal cells. This suggests that determination of presumptive endoderm cells and specificity of zooxanthellae towards presumptive endoderm cells occur earlier in the two *Pocillopora* species than in the soft corals studied.

We described changes in the distribution of zooxanthellae during early development as well as during final maturation of oocytes in the corals *Pocillopora verrucosa* and *P. eydouxi*. Zooxanthellae moved to the hemisphere of the oocyte that contained the germinal vesicle 1 to 2 days before spawning. Zooxanthellae moved to the lateral or basal margins of the surface blastomeres and bulged into extracellular spaces or into the blastocoel. Blastomeres containing zooxanthellae or lipid droplets along with a small amount of cytoplasm were produced, probably by unequal mitotic division, and then dropped into the blastocoel and became endodermal cells. It is not clear whether the presence of zooxanthellae affects development of the blastomere or if the fate of a blastomere is determined by the nature or quantity of its cytoplasm. Further study is necessary to understand how zooxanthellae move to a region of oocytes and to certain areas of blastomeres.

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